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Assessment of the Process Variables for Degradation of Oxalate by *Lactobacillus acidophilus* ATCC 4356 Using Simulated Rumen Fluid Media and Tea

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Abstract

Background and Objective: *Lactobacillus acidophilus* ATCC 4356 was used for oxalate degradation activity. In the present study, for the first time, the simultaneous influence of process variables on the analysis of high oxalate concentration and its optimization in the simulated intestinal environment was performed. In the end, the optimal results were performed in a tea environment as a common oxalate-containing beverage.

Materials and Methods: After screening the design of ten process variables including pH, glucose, sucrose, inulin, ammonium, sodium oxalate, yeast extract, sodium acetate, inoculum age, and size using Plackett-Burman design, a Box-Behnken method was used with four major variables of pH, glucose, sodium oxalate, and inulin

Results and Conclusion: Results showed that oxalate degradation in simulated rumen fluid was significantly affected by pH and sodium oxalate and glucose concentrations. At optimized conditions, oxalate degradation reached 48.94 ±0.98% of initial concentration. Furthermore, oxalate degradation was investigated in tea (as the most common hot drink in many countries such as Iran) at various times, temperature, and glucose concentration. At optimum condition, oxalate concentration reached 98.86% ±1.05 (from 264 to 24 mg per100 ml).

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1. Introduction

Uptake of oxalate (> 45 mg per day) as exogenous (in food) and/or indigenous (synthesized in liver) substance may stimulate kidney stone formation and other health problems linked to high levels of urinary oxalate [1]. The gastrointestinal tract microbiota, such as biodegrading probiotic bacteria, can metabolize oxalate to CO_2 and formate in guts during aerobic growth, while most bacteria cannot use oxalate as a source of energy [2]. *Lactobacillus* (*L.*), *Bifidobacterium* spp. and *Oxalobacter formigenesis* [3] are probiotic bacteria using oxalate [4], both microaerophilic and obligate anaerobic conditions of the human gut, respectively. *L. acidophilus* includes a cluster of genes encoding Oxc and Frc proteins involved in oxalate degradation. The mild acidic condition is the major

requirement for the transcription of the highlighted protein genes [5]. Many bacterial strains (e.g., *L. acidophilus* ATCC 4356) are well characterized for their health beneficial effects in humans in vitro and in vivo [6,7]. To enhance the viability of probiotic cells and promote their growth in human guts, ingredients such as prebiotics are necessary [7]. It is noteworthy that prebiotics can include further helpful effects for the host via binding to toxins. Prebiotic fermentation by gut microbiota produces short-chain fatty acids, which improve the ability of oxalate degradation by *L. paracasei* [8]. Literature reviews have shown that several processes affect oxalate degradation by probiotics [3,9]. Reports have been published on the effects of inulin concentration [10], pH [11], yeast extract [12] and sodium oxalate and ammonium oxalate concentrations [13,14] on the degradation of oxalate. Moreover, reports have demonstrated that oxalate degradation is efficient in the presence of aldohexose [15]. They have demonstrated that high glucose concentrations in culture media may stimulate oxalate metabolism and growth of probiotic bacteria and hence decrease oxalate contents [15].

Gaps in such studies seem to be resulted from a lack of experimental designs to screen and optimize all process variables affecting oxalate degradation in *L. acidophilus*, as solutions for oxalate decreases in high-risk patients as well as other reported health beneficial effects in consumers. However, the reported oxalate degradations are mainly less practical for oxalate concentrations in foods (e.g. 5 and 10 mmol l^{-1} of oxalate) [5,13,14]. Previously, a comprehensive study was carried out by the current authors on oxalate degradation using *Oxalobacter formigenes* DSM 4420 under obligate anaerobic conditions [3]. Results showed improved functions under simulated conditions of gastrointestinal tract.

Therefore, this study aimed to investigate oxalate degradation using *L. acidophilus* as the most common probiotic in high oxalate contents. The *L. acidophilus* ATCC 4356 was selected because it was one of the most effective probiotics for the degradation of oxalate. Results from this experiment were used in another RSM (Response surface methodology) to study the degradation of tea oxalate contents as one of the most common oxalates containing beverages in Asia.

2. Materials and Methods

2.1. Rumen fluid simulated media

Proteose peptone (10 g), yeast extract (5 g), Tween 80 (1 ml), potassium dihydrogen phosphate (2 g), sodium acetate (5 g), di-ammonium hydrogen citrate (2 g), magnesium sulfate heptahydrate (0.05 g), manganese sulfate monohydrate (0.05 g), sodium oxalate and/or ammonium oxalate (10 mmol l^{-1}) and D(+)-glucose anhydrous, and D(+)-sucrose (10 ml each; sterilized using 0.46-µ filters) were dissolved in 500 ml of deionized water. All chemicals were purchased from Merck (Darmstadt, Germany) [14]. This medium was dispensed in 10-ml volumes in culture tubes (16 by 150 mm) under an N_2 gas phase. The final pH of the medium was 6.8.

2.2. Tea media

Briefly, 1 g of tea leaves (Shahrzad, Iran) was infused in 100 ml of hot (90 °C) tapped water at various times of infusion (1, 2 and 3 h), temperatures (50, 75 and 100 °C) and glucose concentrations (0, 20 and 40 g l^{-1}). Tea leaves were removed by filtering the solution through a tea strainer. Tea solution, which contains 264 mg ml^{-1}, was set to room temperature before oxalate analysis.

2.3. Other chemicals and media

De Man-Rogosa-Sharpe (MRS) broth were purchased from Merck (Darmstadt, Germany) and inulin from Beneo (Mannheim, Germany).

2.4. Bacterial preadaptation

Lyophilized cultures of *L. acidophilus* ATCC 4356 were provided by the Iranian Society of Probiotics and Functional Foods (Tehran, Iran). First, *L. acidophilus* ATCC 4356 was pre-adapted to high oxalate contents through anaerobic growth of the bacteria in MRS broth for 16 h and then transferred to MRS broth containing 0.35 mmol l^{-1} of sodium oxalate (pH 5.5 acidified with lactic acid) for 16 h. Growing cells were transferred into MRS broth (pH 5.5) (i) and then into MRS broth (ii) containing 35 mmol l^{-1} of sodium oxalate (pH 5.5) and both and incubated at 37 °C. Pre-adapted *L. acidophilus* was inoculated with 100 µl of the suspension in 1 ml rumen fluid simulated media sterilized at 121 °C for 15 min. The bacterial count of the solution (ii) was carried out through the McFarland standard method and reported as 2×10^8 CFU ml^{-1}.

2.5. Oxalate degradation estimation

Oxalate degradation was calculated using differences between the initial and the final concentrations of the oxalate after overnight incubation (~18 h). After pasteurization of media at 90 ºC for 15 min, the broth was centrifuged at 5000 ×g for 10 min, and the supernatant was used to assess the concentration of the residual oxalate. The control sample was simulated rumen fluid inoculated with *L. acidophilus* without passing incubation time. The oxalate content was assessed using Darman Faraz Kave Kit (Tehran, Iran) in the current study [16]. This kit was based on an enzymatic method, which included oxidation of oxalate by oxalate oxidase followed by the detection of H_2O_2 in the reaction [17]. In this method, one ml of sample was added to 1 ml of dilution solution, and two spoons of active caracole were added to this solution. After 5 min shaking of the solution, it was centrifuged 10 min in 3000 ×g. Then 50

µl from the clear layer was sampled and 3 tubes with the mentioned structure were prepared (Table 1).

These mixtures were gently mixed and kept at 37 °C for 10 min. Then the absorbance of T and S tubes should be read in front of tube B at 590 nm wavelength. Calculation of oxalate concentration obtained from the Eq. 1:

Oxalate concentration (mmol l^{-1})= (absorbance of T tube/S tube)×0.25×2×dilution coefficient (Eq.1)

2.7. Experimental design and selecting variables

The PBD is an excellent screening method due to its ability to simultaneously investigate effects of a large number of process variables in biotechnological research [18,19]. This design was used to efficiently select important simulated rumen fluid medium components affecting the oxalate degradation activity by *L. acidophilus*. This will provide as a guide in developing an effective medium composition for enhanced oxalate degradation. The RSM involves major, and interaction effects described as curves and enhance optimal process settings [20,21]. Variables of the screening step (at two levels) included pH, sucrose, glucose, sodium oxalate, ammonium oxalate, and inulin concentrations, yeast extract, inoculum size, age, and sodium acetate, which were selected based on the previous reports (as shown in Table 2) (Subtitle 2.8). As shown in Table 2, maximum oxalate degradation (19.20 % ±0.60) resulted in trial Number 10. After determining of four main variables in PBD (pH, sodium oxalate, inulin, and glucose), oxalate degradation activity was optimized by RSM. Then obtained optimized condition for oxalate degradation in simulated rumen fluid media, was again applied for oxalate degradation in tea through Box-Behnken design. The variables included time, temperature, and glucose at three levels. The variables and their levels for PBD and RSM are described in Tables 2 and 3.

2.7. Selection of variables and range finding

2.7.1. pH

In general, pH is an important factor to increase the bacterial oxalate degradation activity through enhancement of the bacterial Frc and ogc gene expression [11]. In this study, the pH of the samples (pH: 5.5-7) was adjusted to certain values using 1 N of either HCl or NaOH solution. The pH of rumen fluid simulated media was adjusted to 5.5-7. In PBD two levels of pH (5.5 and 7) and RSM design three levels of pH (5.5, 6 and 6.5) have been considered (Tables 2 & 3).

2.7.2. Glucose and sucrose concentrations

The concentration of glucose (20 and 40 g l^{-1}) and sucrose (10 and 20 g l^{-1}) was also selected as variables in screening design. Literature review and pre-experiences show that addition of glucose and sucrose seems to be essential for *L. acidophilus* oxalate degrading activity since sugars are the most common carbon sources for *L. acidophilus* [15]. Levels of Sucrose in PBD were (10 and 20 g l^{-1}), levels of glucose in PBD were (20 and 40 g l^{-1}), and RSM design glucose levels were (35, 40, and 45 g l^{-1}) (Table 2, 3).

2.7.3. Sodium oxalate and ammonium oxalate concentrations

The concentration of sodium oxalate (5-25 mmol l^{-1}) and ammonium oxalate (10 and 20 mmol l^{-1}) could influence oxalate degradation efficiency. It has been shown that in the presence of 10 mmol l^{-1} of ammonium oxalate, the *L. acidophilus* growth increased but decreased at further concentrations of ammonium oxalate at 20 mmol l^{-1} [14]. Sodium oxalate concentration levels in PBD were 5 and 20 mmol l^{-1}, and in RSM design, its levels were 5, 15, and 25 mmol l^{-1} (Table 2, 3).

2.7.4. Yeast extract

Yeast extract is an additional nitrogen source for bacterial growth, which its possible impact was studied. The range we have used for yeast extract is 0-1 percent in the medium. In the absence of yeast extract, *L. acidophilus* is not able to grow [12] (Tables 2 & 3).

2.7.5. Inoculum age

In this research, inoculum age of 18 and 36 h was applied for bacterial inoculation, due to another pre-experience which indicates that 36 h inoculum age caused a higher production rate (Table 2) [22].

2.7.6. Inoculum size

Two bacteria inoculations were used in PBD studies, including 10^{10} CFU ml^{-1} as maximum and 10^{6} CFU ml^{-1} as the minimum bacterial concentrations according to Iranian probiotic monitoring protocols (Table 2).

2.7.7. Inulin

Inulin (0-1.5 g l^{-1}) was selected as another process variable in this study because to assess its effect on the growth of probiotic bacteria. Gut microbiota could metabolize them and produces short-chain fatty acids, which, on behalf of itself, improves the capability of survival and activity of the gut probiotic bacteria (Table 2,3).

2.7.8 Sodium acetate

Sodium acetate is one of the primary substrates in simulated fluid media. It can alleviate the acidic pH of the media and inhibit the formation of calcium oxalate crystals.

2.8. Statistical analysis

In this study, PBD and RSM methodologies were carried out using Design Expert Software v.11.0.3.0 (Stat-Ease, USA). All experiments were carried out in triplicate.

3. Results and Discussion

3.1. Regression model and statistical testing

The PBD and response surface design were used to analyze major and interaction effects of the media pH, glucose, sucrose, sodium oxalate, ammonium oxalate, inulin and yeast extract concentrations, and inoculum size and age. Figure 1 shows the percentage contribution of the rumen fluid simulated components. The results which were derived from expert design software revealed that pH and inulin are the most contributing components with 30%. Thus, pH (effective factor in oxalate degradation gene expression) and inulin (as growth promotor for *L. acidophilus*) has been reported to act as an inducer during oxalate degradation, owing to their potential in increasing bacterial growth and oxalate degradation activity and the optimum condition was achieved for oxalate degradation. Multiple regression analysis was carried out to fit the best model for each response. Correlation coefficient and lack of fit test were used to show the significances of the regression equation models (R^2 = 92.17 ±0.01%). Analysis of variance (ANOVA), regression coefficients of individual linear or quadratic model, and optimization of the polynomials were reported at $p \leq 0.01$ and $p \leq 0.05$.

3.1.1. PBD for screening the main variables for oxalate degradation in simulated rumen fluid

The linear equation of oxalate degradation ($p \leq 0.05$) was derived using PBD as Eq. 2:

$$Y = -4.8A - 0.4C + 0.5D + 0.4H - 0.1AC + 0.1D + 0.1 \quad \text{Eq. 2}$$

Where, Y was the response representing oxalate degradation efficiency (%) and A, C, D and H were respectively coded terms for pH (A), sodium oxalate (C), glucose (D) and inulin (H) four-test variables. The ANOVA results and statistical plots showed acceptability of this model. In this study, A (p= 0.0005), C (p= 0.0047), D (p= 0.0061) and H (p= 0.0037) were significant model terms. Increases in A and C negatively affected oxalate degradation activity (R^2= 92.17 ±0.01%, Adjusted R^2= 89.19%, Mean square= 108.8).

3.1.2. RSM for the optimization of oxalate degradation in simulated rumen fluid

The full quadratic equation of oxalate degradation ($P \leq 0.01$) was derived using RSM as Eq. 3:

$$Y = +39.19 - 4.82A - 0.5412B + 3.20C - 4.32D - 16.67AC + 5.42AD - 7.69A^2 - 14.56B^2 - 12.54C^2 - 9.56D^2 \quad \text{Eq.3}$$

Where, Y was the response representing oxalate degradation efficiency (%) and A, B, C and D were respectively coded terms for pH (A), inulin (B), sodium oxalate (C) and glucose (D) three-test variables. The ANOVA results and statistical calculation showed acceptability of this model. In this study, A (p= 0.00262), D (p= 0.0141), AC (p= 0.01), A^2 (p= 0.0064), B^2 (p= 0.006), C^2 (p= 0.001) and D^2 (p= 0.004) were significant model terms. Increasing in A and D can negatively affected oxalate degradation activity (R^2= 92.17%, adjusted R^2= 84.35%).

3.2. Major and interaction effects of the process variables affecting oxalate degradation

3.2.1. Effects of pH

As shown in Tables 2 and 3, a correlation was reported between the pH of media and bacterial oxalate degradation. The values of the *t*-test through the Pareto chart in PBD design shows that increased pH negatively affected oxalate degradation (Figure 2). The results of RSM showed that at pH 5.5 and 0.98 g l^{-1} of inulin, sodium oxalate was metabolized by the bacteria at a high concentration of 22.79 mmol l^{-1} (Figures 3A and 3B). These results were similar to results by Azcarate-Peril et al, who found that transcription of the *L. acidophilus* NCFM *oxc* and *frc* genes needed acid induction at pH 5.5 [11]. In PBD (Table 2), the maximum oxalate degradation is in experiment number 10 with a higher pH of 7. In this condition, a Cd/Mn transport ATPase gene is more active and leading to more oxalate degradation activity [14]. In RSM design (Table 3), the highest oxalate degradation is in pH 5.5, which is the optimum condition for Frc and ogc gene expression.

3.2.2. Effects of sugar concentration

The highest oxalate degradation rate of *L. acidophilus* has resulted from 37.47 g l^{-1} of glucose in rumen fluid simulated media (Figure 3C), [14]. They examined sugar concentrations of 40-50 g l^{-1} in rumen fluid simulated media and verified that sugars were essential for the bacterial oxalate degradation. In the current study, experiments on sucrose using *t*-test analysis showed no significant effects on *L. acidophilus* oxalate degradation (Figure 2). In both PBD and RSM design, the highest oxalate degradation activity has resulted from the highest glucose and sucrose concentration (Table 2, 3).

Table 1. Sample preparation for oxalate measurement by kit

Content of tube	B (µl)	S (µl)	T (µl)
Sample	-	-	50
Standard solution	-	50	-
Distilled water	50	-	-
Coloring reagent	1000	1000	1000
Enzymatic solution	250	250	250

Table 2. Treatment combinations and responses for the assessment of variables affecting oxalate degradation in rumen fluid simulated media using Plackett-Burman design

Trial No.	A: pH	B: Sucrose /g l^{-1}	C: Glucose /g l^{-1}	D: Na oxalate /mmol l^{-1}	E: NH_4oxalate /mmol l^{-1}	F: Yeast extract %	G: Inoculum /CFU l^{-1}	H: Inulin /g l^{-1}	J: Inoculum age/ h	K:Na acetate /mmol l^{-1}	Oxalate degradation %
1	5.5	20	40	20	10	1	10^6	0	18	10	7.33 ±0.03
2	5.5	10	40	20	20	0	10^6	1	36	20	1.92 ±0.88
3	5.5	20	20	5	20	1	10^6	0	36	10	6.50 ±0.70
4	7	10	20	5	20	0	10^{10}	0	36	10	4.62 ±0.91
5	7	10	40	20	10	0	10^{10}	0	18	10	11.33 ±0.05
6	7	10	20	20	20	1	10^6	1	18	10	10.18 ±0.75
7	5.5	20	20	20	20	0	10^{10}	1	18	10	2.64 ±0.05
8	7	20	20	5	10	1	10^{10}	1	18	20	13.75 ±0.49
9	7	10	40	5	10	1	10^6	1	36	10	12.50 ±0.80
10	7	20	40	5	20	0	10^6	0	18	20	19.20 ±0.60
11	7	20	40	20	20	1	10^{10}	0	36	20	5.66 ±0.45
12	7	20	20	20	10	0	10^6	1	36	20	0.01 ±0.00
13	5.5	10	20	20	10	1	10^{10}	0	36	20	1.60 ±0.03
14	5.5	20	40	5	10	0	10^{10}	0	36	10	0.02 ±0.00
15	5.5	10	20	5	10	0	10^6	1	18	20	4.16 ±0.03
16	5.5	10	40	5	20	1	10^{10}	1	18	20	9.20 ±0.70

3.2.2. Effects of sugar concentration

The highest oxalate degradation rate of *L. acidophilus* has resulted from 37.47 g l^{-1} of glucose in rumen fluid simulated media (Figure 3C), [14]. They examined sugar concentrations of 40-50 g l^{-1} in rumen fluid simulated media and verified that sugars were essential for the bacterial oxalate degradation. In the current study, experiments on sucrose using *t*-test analysis showed no significant effects on *L. acidophilus* oxalate degradation (Figure 2). In both PBD and RSM design, the highest oxalate degradation activity has resulted from the highest glucose and sucrose concentration (Table 2, 3).

3.2.3. Effects of oxalate concentration

Oxalate contents of the media generally affect bacterial oxalate degradation activity. Typically, *L. acidophilus* utilizes oxalate, not as the primary source of carbon; hence, enhancement variables such as pH, inulin, and glucose concentrations should be used in media to induce the bacterial oxalate degradation activity. Decreased pH level at 5.5 and presence of inulin and glucose at 0.98 and 37.46 g l^{-1} can respectively increase oxalate utilization at a high concentration (22.97 mmol l^{-1}) (Figures 3B and 3D). The highest level of oxalate degradation in RSM was 48.94% ±0.98, which was higher than that in control samples (15% ±1). As shown in the Pareto chart, the sodium oxalate concentration positively affected oxalate degradation. Increased oxalate degradation could be due to bacterial preadaptation of oxalate and other variables that helped the bacteria degrading further oxalate. The quadratic model for oxalate degradation was statistically significant ($p \leq 0.01$), but the lack of fit was insignificant relative to the pure error. The oxalate degradation results for the 29 treatments are shown in Table 3.

A high concentration of oxalate would be toxic to bacterial cells. Therefore as shown in Table 2, trials number 2, 7, and 11 had low oxalate degradation activity.

3.2.4. Effects of yeast extract concentration

Results of PBD showed that yeast extract included a great effect on oxalate degradation at 0.1% w v^{-1} ($p \leq 0.01$), while further increased concentration of the yeast extract included a negative effect on the bacterial oxalate degradation (Figure 2). This result is due to an excess source of nitrogen, which is not consumed with bacteria. Otherwise, in low concentrations of yeast extract, if there is not enough nitrogen, the bacterial oxalate degradation may decrease (such as trial numbers 12 and 14 in Table 2).

3.2.5. Effects of inoculum age

The current PBD *t*-value results demonstrated that inoculum age resulted no significant impact on oxalate degradation (Figure 2). Since both selected ages for inoculum are in the duration of logarithmic growth phase of bacteria, there has not been any significant difference between 18 h and 36 h inoculum ages.

3.2.6. Effects of inoculum size

As a result of *t*-value, inoculum size at two considering levels showed no significant impact on oxalate degradation (Figure 2). Regarding the standard probiotic population, both considering levels are in the acceptable range of probiotic cell counts. However, the oxalate degradation of probiotics did not show any significant difference.

3.2.7. Effects of inulin concentration

Response surface generated from the second-order coefficients showed a close correlation with the substrate use (Figures 3D and 3E). Results of the *t*-test demonstrated

that inulin positively affected bacterial oxalate degradation (Figure 2). This verified that the production of short-chain fatty acids from inulin fermentation was closely associated with the substrates [8]. Thus, it is suggested that an increase in sodium oxalate usage in experimental investigations may generate higher concentrations of hydrolysis products and subsequently needs further short-chain fatty acids productions. As results of optimum condition in RSM showed, the best concentration of inulin is 1 g l^{-1}, which consequently led to more oxalate degradation activity (trials number 2, 3, 7, 25, and 27 of RSM design) (Table 3).

Table 3. Treatment combinations and responses for the assessment of variables affecting oxalate degradation in rumen fluid simulated media using response surface method

Run	A: pH	B: Inulin (g l^{-1})	C: Sodium oxalate (mmol l^{-1})	D: Glucose (g l^{-1})	Oxalate degradation (%)	Bacterial final population (CFU ml^{-1}) × 10^6
1	6	1.5	5	40	8.75 ±0.65	20
2	6	1	15	40	35.58 ±0.98	220
3	6	1	15	40	37.42 ±1.01	120
4	6.5	0.5	15	40	11.23 ±0.87	150
5	6.5	1	15	45	12.57 ±0.75	50
6	6	0.5	15	45	16.57 ±0.65	170
7	6	1	15	40	36.85 ±0.87	200
8	6	0.5	15	35	15.14 ±0.74	36
9	5.5	1.5	15	40	12.67 ±0.62	20
10	6	1	5	45	11.93 ±0.57	170
11	6.5	1	15	35	21.42 ±0.87	220
12	6	1	25	35	18.56 ±0.66	140
13	6	0.5	25	40	12.22 ±0.62	160
14	6.5	1	5	40	26.36 ±0.88	230
15	6.5	1	25	40	3.78 ±0.054	70
16	6	1.5	25	40	15.33 ±0.38	230
17	6	1	5	35	11.83 ±0.61	200
18	5.5	1	5	40	4.84 ±0.053	52
19	6	1	25	45	16.66 ±0.62	9
20	6	1.5	15	45	12.28 ±0.75	110
21	5.5	1	15	45	12.28 ±0.66	170
22	5.5	0.5	15	40	23.10 ±0.90	200
23	6.5	0.5	15	40	12.50 ±0.70	15
24	6	1.5	15	35	24.38 ±0.95	150
25	5.5	1	25	40	48.94 ±0.98	190
26	5.5	1	15	35	42.81 ±1.05	170
27	6	1	15	40	45.57 ±0.98	130
28	6	0.5	5	40	13.44 ±0.56	100
29	6	1	15	40	40.55 ±0.88	140

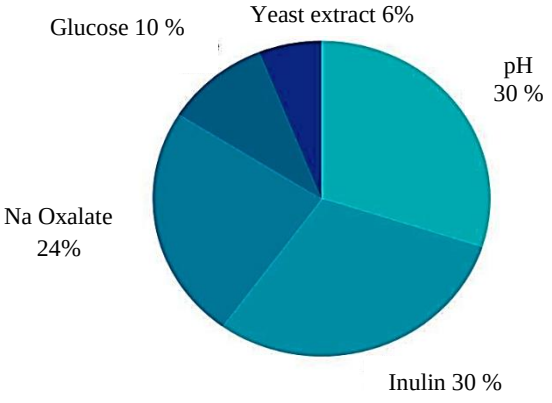

Figure 1. Pie chart of variables contribution in optimizing the oxalate degradation by *Lactobacillus acidophilus*

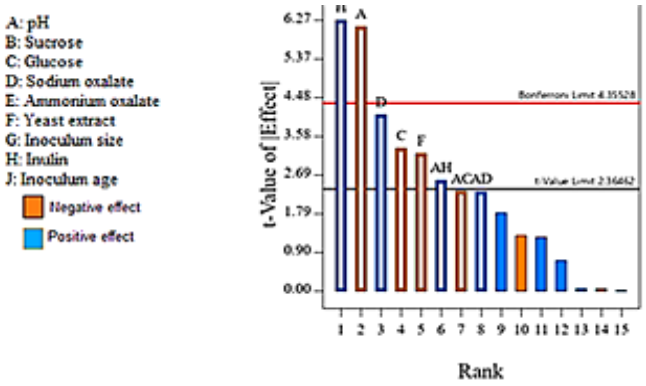

Figure 2. Pareto Chart of variables in in optimizing the oxalate degradation by *Lactobacillus acidophilus*

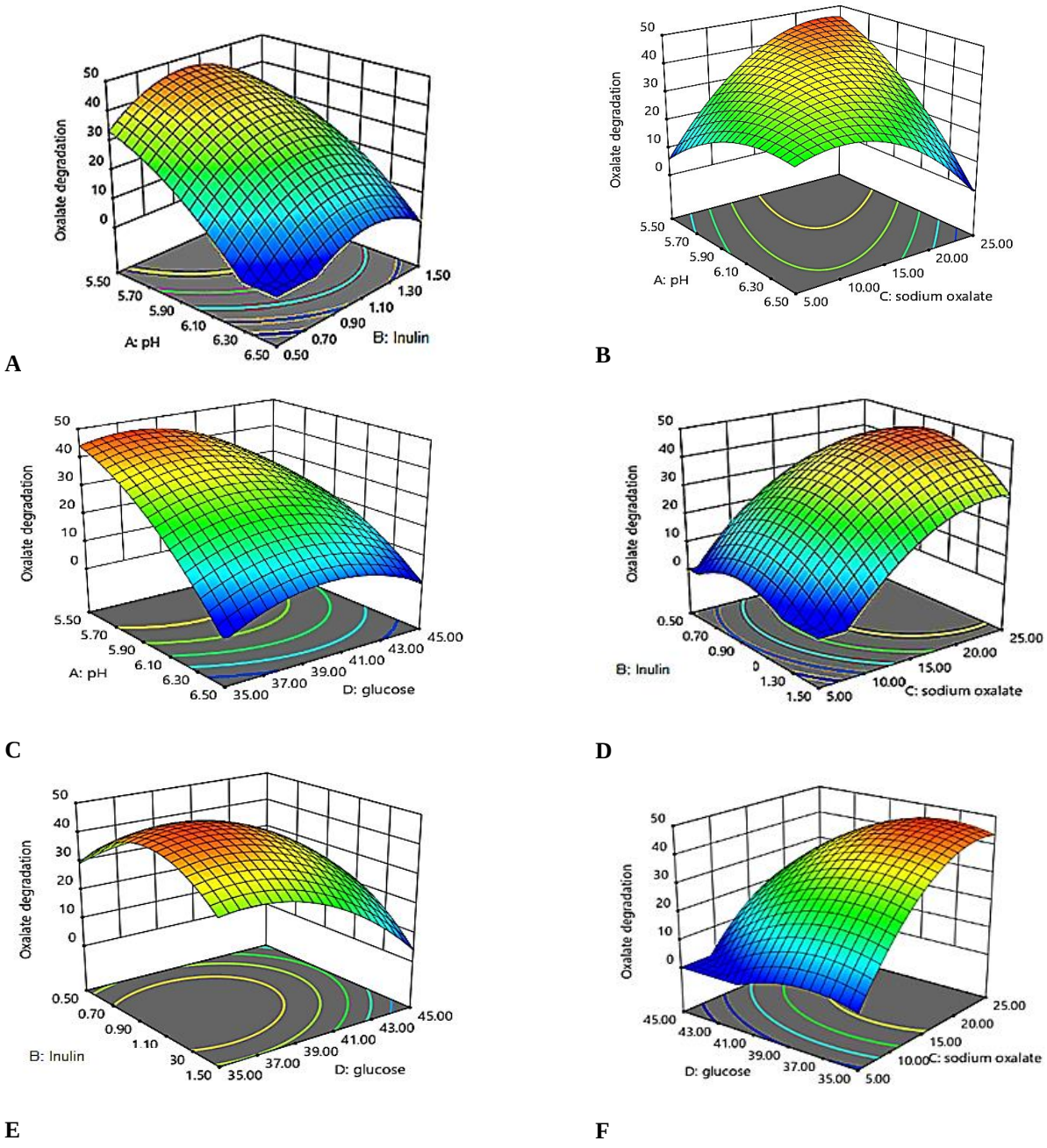

Figure 3. Response surface for impact of process variables including pH at 6.5 (A), inulin at 1.48 (g l^{-1}) (B), ammonium oxalate at 16.04 (mmol l^{-1}) (C) and glucose at 44.82 (g l^{-1}) (D) on oxalate degradation

3.3. RSM for the optimization of oxalate degradation in tea

Oxalate degradation was investigated in tea (oxalate content about 12 mmol l^{-1}) as the most common hot drink in many Asian countries such as Iran, based on the results of optimum conditions in simulated rumen fluid. Likewise, the optimum pH for *L. acidophilus* oxalate degradation and oxalate oxc gene expression is in the acidic condition we considered black tea which its pH is 5-5.5. Additionally, we considered glucose, which is necessary for bacterial oxalate degradation, and it concluded from our results on simulated rumen fluid. Time and temperature are two important parameters in brewing tea, so we should investigate these parameters (Table 4). As shown in Figure 3A, 3B, 3F the optimum points for oxalate degradation in the tea samples are included 1.74 h, 54.20 °C, and 12 g l^{-1} of glucose concentration. At optimum conditions, the oxalate content reached to 98.86 ±0.87% (from 264 to 41 mg per 100 ml).

Previous studies reported 0.94 and 0.73% oxalate degrading activities after three days of incubation with *L. brevis* in 10 and 20 mM ammonium oxalate media, respectively [23]. Other studies found oxalate degrading activities ranging from 54.8-58.3% in the fermentation of 10 mM of potassium oxalate with *L. fermentum* TY5, TY12,

and AM3 strains [14]. In similar studies, 20 and 47% degrading activities were found in the fermentation of 5 mmol l^{-1} of sodium oxalate with *L. rhamnosus* PB41 and PB45 strains [24]. In general, oxalate-degrading activity in this study was higher than the previous studies. In terms of differences within-species, oxalate degradation rates of the same strain were variable at different concentrations of oxalate. Differences with-in-species or within-strains in oxalate degrading activity could be explained with cells' physiological states of the cells [24], acidic conditions, and pre-adaption of cells with low levels of oxalate.

Our investigations indicated the synergistic effect of synbiotic (mixed culture and inulin) on oxalate degrading activity. Co-culture of *L. acidophilus* with *Oxalobacter formigenes* may enhance the oxalate degradation efficiency since limited information has been reported on the influence of mixed culture on oxalate biodegradation. In a study, the mixture of *L. casei* and *Bifidobacterium breve* has a lowering effect upon urinary oxalate excretion in stone-forming subjects [25]. The difference in our study compared to the previous reports is addition of inulin to rumen fluid media which enhanced the degrading activity. As oxalate degradation may be influenced by many factors, especially in vivo, further studies are required to identify a suitable condition for oxalate degradation in the gastrointestinal tract. Considering various effective parameters would enhance the bacterial ability for oxalate degradation especially in high oxalate content media.

In this study, after preadaptation of the bacterium, a comprehensive screening design was carried out for the first time on ten possible effective variables on oxalate biodegradation. Then, Box-Behnken design was used at a various range for the major effects of pH (5.5-7), glucose (35-45 g l^{-1}), sodium oxalate (5-25 mmol l^{-1}) and inulin (0.5-1.5 g l^{-1}). Oxalate degradation was significantly affected by the pH and inulin and sodium oxalate and glucose concentrations. Results from RSM showed that the addition of 0.987 g l^{-1} of inulin and 37.467 g l^{-1} of glucose at pH 5.5 could improve the desirability of optimization results up to 90%. These results were then used for the assessment of oxalate degradation in tea-a high oxalate, acidic and popular drinking- at various times (1, 2 and 3 h), temperatures (50, 75 and 100 °C) and glucose concentrations (0, 20 and 40 g l^{-1}). At optimum conditions, oxalate contents decreased up to 98.86% ±0.87, and it will be a hopeful finding for the diet of individuals who are suffering from hyperoxaluria.

4. Conclusion

In the current study, oxalate degradation by *L. acidophilus* ATCC 4356 has been studied at high oxalate concentrations. Preadaptation tests in sequence ascending cultures showed significant increases in bacterial tolerance and growth in the presence of oxalate from 0.35 to 35 mmol l^{-1}. Then, a screening design was carried out on ten process variables, including pH, glucose, sucrose, inulin, ammonium, sodium oxalate, yeast extract, sodium acetate, inoculum age, and size. The screening design demonstrated the main variables (pH, glucose, inulin, and sodium oxalate) on oxalate biodegradation. Then, Box-Behnken design was used for pH 5.5-7, glucose 35-45 g l^{-1}, sodium oxalate 5-25 mmol l^{-1}, and inulin 0.5-1.5 g l^{-1}) to optimized condition. The results of optimization step showed that the addition of 0.98 g l^{-1} of inulin and 37.46 g l^{-1} of glucose at pH 5.5 could improve desirability up to 90%.

Oxalate-degrading activity in this study was higher than the previous studies due to our experimental design, including screening and optimization steps. Based on the obtained results and better oxalate degradation in acidic pH, in the final step, the results were used in acidic food containing oxalate. These results were then used to assess oxalate degradation in tea, which included high concentrations of oxalate at various times, temperatures, and glucose concentrations. Again optimized conditions were obtained in tea, and oxalate content decreased up to 98.86% ±0.87. In conclusion, findings of the current study can be used to develop low oxalate plant foods with optimum qualities at commercial scales with considering effective variables at their optimum concentrations, which were resulted from this research.

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6. Conflict of Interest

The authors declare no conflict of interest.

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ارزیابی متغیرهای موثر بر فرآیند تجزیه اگزالات توسط *لاکتوباسیلوس اسیدوفیلوس* ATCC 4356 در محیط شبیه‌سازی شده معده-روده و محیط چای

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چکیده

سابقه و هدف: فعالیت تجزیه اگزالات باکتری *لاکتوباسیلوس اسیدوفیلوس* ATCC 4356 مورد مطالعه قرار گرفت. در مطالعه حاضر، برای اولین بار، تأثیر همزمان متغیرهای فرآیند در تجزیه و تحلیل غلظت اگزالات بالا و بهینه‌سازی آن در محیط شبیه‌سازی شده معده-روده انجام شد. در پایان، نتایج بهینه در یک محیط چای به عنوان یک نوشیدنی حاوی اگزالات انجام شد.

مواد و روش ها: با استفاده از غربالگری به وسیله طراحی پلاکت برمن متغیرهای فرآیند شامل pH، گلوکز، ساکارز، اینولین، آمونیوم، اگزالات سدیم، عصاره مخمر، استات سدیم، سن تلقیح و اندازه تلقیح مورد بررسی قرار گرفتند. سپس با استفاده از طراحی باکس بنکن جهت بهینه‌سازی فرایند چهار متغیر اصلی pH، گلوکز، سدیم اگزالات و اینولین مورد بررسی قرار گرفتند.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که تجزیه اگزالات در محیط شبیه‌سازی شده روده به طور قابل توجهی تحت تأثیر pH و سدیم اگزالات و غلظت گلوکز قرار دارد. در شرایط بهینه تجزیه اگزالات، غلظت انتهایی آن به ۰/۹۸ ±۹۸/۸۶ درصد غلظت اولیه رسید. علاوه بر این، تجزیه اگزالات در چای (به عنوان رایج‌ترین نوشیدنی گرم در بسیاری از کشورها از جمله ایران) در زمان‌های مختلف، دما و غلظت گلوکز مورد بررسی قرار گرفت. در شرایط مطلوب، غلظت اگزالات با ۱/۰۵± ۸۶ /۹۸ درصد کاهش از ۲۶۴ تا ۲۴ میلی‌گرم در ۱۰۰ میلی‌لیتر رسید.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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-تجزیه زیستی
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-اگزالات
-طراحی پلاکت برمن
-روش سطح پاسخ

***نویسنده مسئول**

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